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An N-End Rule Destabilization Mutant Reveals Pre-Golgi Requirements for Sec7p in Yeast Membrane Traffic

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Sec7 protein (Sec7p) is required for membrane traffic in the yeast secretory pathway. Because Sec7p regulates more than one stage in the pathway, it has been difficult to assign the most proximal requirement for Sec7p action. We have engineered a novel mutant whose Sec7p levels are regulated by growth conditions and by selective protein destabilization according to the N-end rule. Sec7p depletion causes cell growth arrest and accumulation of transport proteins with post-translational modifications indicative of Sec7p dependence for ER-to-Golgi traffic, in addition to the already characterized Golgi requirements. Immuno-EM of *sec7* revealed exaggeration of ER and Golgi membranes with protein accumulation in these exaggerated structures, suggesting that these regions may represent staging areas for cargo sorting and vesicle assembly.

Key Words: **Sec7p, yeast, secretory pathway, endoplasmic reticulum, Golgi apparatus.**

Membrane traffic between organelles of the secretory pathway is mediated by transport vesicles that function in the sorting, targeting and delivery of cargo. The study of the proteins responsible for yeast membrane traffic has been facilitated by the genetic selection of temperature-sensitive secretory mutants (1, 2). This collection includes coat proteins recruited from the cytosol during transport vesicle formation (3). One of these proteins, Sec7p, is an abundant 230 kDa phosphoprotein that is recruited onto membranes for traffic at multiple stages of the pathway (4–7). Shifting temperature-sensitive (*sec7*^{ts}) mutant yeast to the restrictive temperature resulted in the accumulation of cargo proteins at each stage where Sec7p function was required (8, 9). EM images of these mutants showed exaggerated stacks of membranes resembling the Golgi apparatus in plant and animal cells (10–12). Immunofluorescence data, like the EM results, were interpreted to mean that Sec7p function was restricted to the Golgi apparatus, with no apparent involvement in the ER-to-Golgi stages of the pathway (4, 10, 13). However, data from transport assays demonstrated a role for Sec7p in ER-to-Golgi traffic *in vitro* (14, 15). If Sec7p is part of the machinery for regulating membrane traffic between compartments, then it is reasonable to postulate its involvement at each stage from the ER and through the Golgi apparatus.

To analyze the role of Sec7p more precisely, we constructed a conditional mutant strain where Sec7p expression can be regulated, and was further engineered for destabilization according to the N-end rule, thus speeding its turnover rate *in vivo* (16). We show that the presence of a "destabilizing" residue at the amino-terminus exerted a dramatic effect on the steady state abundance and turnover rate of the usually long-lived Sec7p. Using this strain, we utilized biochemical and morphological approaches to define the most proximal requirement for Sec7p function in the secretory pathway.

MATERIALS AND METHODS

Plasmids, strains, media and antisera. The wild-type yeast used to construct the regulated *SEC7* strain was AFY71 (W303 strain, 17). The *sec7*^{ts} strains used in this study were AFY80, *sec7-1* or AFY83, *sec7-4* (9). Plasmid amplifications and subcloning was carried out by standard techniques, using *E. coli* HB101 strain. YNB minimal medium was composed of 6.7 g/l yeast nitrogen base without amino acids (Difco), 2% glucose or 2% galactose plus 2% raffinose, and supplemented with amino acids as appropriate. Antisera to Sec7p (4), glucose-6-phosphate dehydrogenase (Sigma), invertase (9) and CPY (9) were used. Gas1p and affinity-purified HDEL antisera were kindly provided by Drs. Fankhauser (18) and Pelham (19) respectively.

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Generation of the conditional-lethal destabilizing sec7 mutant. To facilitate rapid turnover of Sec7p, the *SEC7* gene was engineered into a destabilizing fusion gene construct as described previously (20). This construction fused ubiquitin, a signature destabilizing amino acid of the N-end rule, arginine, followed by an influenza epitope tag, all in frame with the full-length *SEC7* gene (see Fig. 1). To generate this fusion construct, the *SEC7* coding region was first cloned into pGEMflu (20). From this construct, the LacI/flu/SEC7 sequences were cloned into the pSE362 expression vector with the *GAL10* promoter and ubiquitin/destabilizing Arg residue from pUB23 to create the plasmid pGAL-*ArgSEC7*. The fusion protein expressed from this construct was termed Arg-Sec7p. A fusion protein with a stabilizing Met residue was similarly engineered. To generate the pGAL-SEC7 mutant strain, wild type AFY71 yeast bearing pGAL-*ArgSEC7* were transformed with a plasmid to disrupt the genomic copy of *SEC7*. To prevent recombination with the plasmid-borne *SEC7*, the *LEU2* gene replaced much of the 5′ noncoding and coding regions of *SEC7*. This disrupt construct could not recombine with pGAL-*ArgSEC7*, which contained only the *SEC7* coding region. Transformants were replica-plated onto media containing glucose and colonies that grew on galactose were isolated.

Immunoblot analysis. Cells were ruptured by glass beads with a urea-SDS lysis buffer (21). Lysates were heated at 55°C 30 minutes, then diluted with SDS-sample buffer. Proteins were resolved on 8% polyacrylamide SDS-gels, then transferred to nitrocellulose overnight. Antibody binding to the nitrocellulose was detected with ^{125}I-protein A (ICN), and the blots were exposed to film at −80°C. The immunoblots were quantitated by PhosphorImager (Molecular Dynamics).

Cell radiolabeling and immunoprecipitations. Whole yeast cells were radiolabeled in YNB media with Tran^{35}S-label (ICN) at 50 μCi per OD_{600} (10^7) cells, as described previously (21). The radiolabel pulse was performed for 7 min at 30°C (or 37°C for ts mutants), then chased with a 100× dilution of 0.4% methionine, 0.3% cysteine in 50 mM NH_2SO_4. Lysates were prepared in 8M urea, 1% SDS. Aliquots were mixed with antisera plus protein A-Sepharose beads (Pharmacia) diluted in immunoprecipitation buffer. The immunoprecipitated proteins were dissociated in urea-SDS lysis buffer for 10 min at 70°C, before dilution with SDS sample buffer for resolution on 8% polyacrylamide gels. Use of fresh 33% acrylamide/0.5% bis-acrylamide stock solution improved electrophoretic resolution of the CPY intermediates.

Electron microscopy. The wild-type or *sec7-4*^{ts} mutant AFY83 yeast were grown overnight in YP + 5% glucose at 25°C, washed briefly then transferred to YP + 0.1% glucose at 37°C for 1.5h. Cells were fixed in 3% paraformaldehyde, 0.5% glutaraldehyde. Preparation of the cells for embedding in Lowicryl HM20, sectioning and immuno-labeling was described (22).

RESULTS

Engineering a destabilized yeast sec7 mutant. To assess the effects of Sec7p depletion on the function of the secretory pathway *in vivo,* we constructed a strain in which the genomic copy of *SEC7* was disrupted and substituted by a plasmid-borne copy under the control of a regulatable *GAL10* promoter. We experienced problems with depleting Sec7p from the cell if the wild type gene was under galactose promoter control. Due to the long halflife of wild type Sec7p (>2h, data not shown) and 6-fold overexpression of the *SEC7* gene product in galactose, cells shifted to glucose required over 24 h before the growth rate of the culture slowed. Sec7p levels decreased primarily due to cell division, and phenotypic defects eventually appeared even though Sec7p pools were never fully depleted. Therefore, to improve the efficiency of protein depletion after promoter repression, a strategy was developed to destabilize Sec7p according to the N-end rule (16, 20). Ubiquitinated fusion proteins are rapidly processed in yeast cells by a deubiquitinating enzyme to yield free ubiquitin plus the remainder of the fusion protein. This reaction can be exploited to generate proteins that differ in their N-terminal amino acid which, in turn, can determine the stability of the protein. According to the N-end rule, methionine is a stabilizing residue, while arginine is a destabilizing amino acid at the protein amino-terminus. The construct pGAL-*ArgSEC7* expressed the Arg-Sec7p fusion protein under control of a galactose promoter (*GAL10*), which is induced in media containing galactose, yet repressed when glucose is present (Fig. 1). Another Sec7p fusion protein was engineered with N-terminal methionine (pGAL-*MetSEC7*). The Sec7p fusion proteins were competent for function, as their expression suppressed temperature-sensitive defects in *sec7*^{ts} mutants at 37°C (data not shown). A pGAL-SEC7 mutant was generated by disrupting the *SEC7* chromosomal gene in wild-type yeast harboring pGAL-*ArgSEC7*, making Arg-Sec7p the sole *SEC7* gene product in this strain.

Sec7p depletion leads to growth arrest. Replica-plating the pGAL-SEC7 strain from galactose to glucose confirmed that Sec7p was essential to cell growth (23). Growth in liquid media was monitored for the kinetics of growth arrest after switch to glucose. pGAL-SEC7 cells grown in galactose showed similar doubling times (∼2.7h) as wild-type yeast (Fig. 1B). Surprisingly, growth arrest of pGAL-SEC7 yeast in glucose was not observed until 14h (∼5 doublings). However, the pGAL-SEC7 cells were viable for up to 19h of glucose repression. To demonstrate cell viability, equal aliquots from wild-type or pGAL-SEC7 cultures shifted to glucose were plated onto galactose. At 16-19h, similar numbers of colonies arose from both cultures (Fig. 1C). Yet after 21 h on glucose, the pGAL-SEC7 cells were mostly inviable. As expected, pGAL-SEC7 cells plated onto glucose at any time point did not form colonies. Hence, for a significant window of time, Sec7p depletion was not lethal. This result emphasized that for up to 19h in glucose, the observed phenotypes were not due to cell death, but reflected Sec7p requirements.

Sec7p levels were examined by immunoblots of lysates from cells harvested at different times after switching to glucose/repressing medium and quantitated by normalizing the blots to endogenous levels of glucose-6-phosphate dehydrogenase. Cells expressing pGAL-*MetSEC7* (Fig. 2A) or normal SEC7 under galactose promoter control (Fig. 2B) exhibited ∼6-8 fold higher Sec7p levels than wild type yeast. In contrast, cells expressing pGAL-*ArgSEC7* in galactose showed only ∼20% Sec7p levels com-

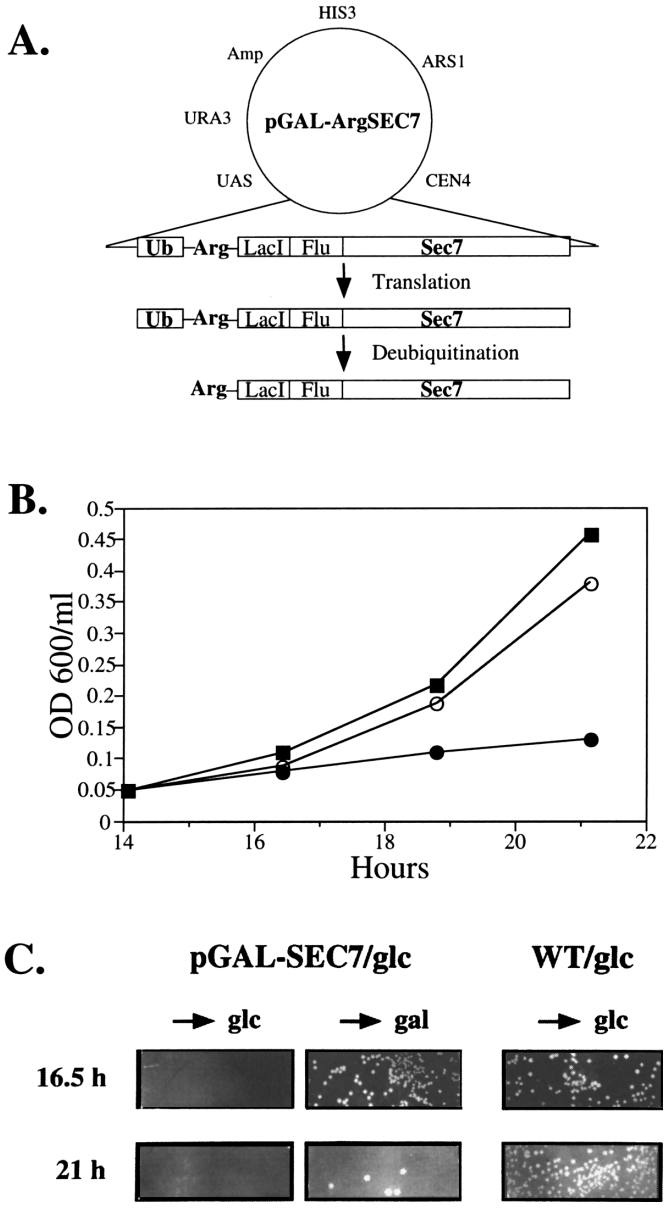

FIG. 1. Sec7p shut-off affects cell growth. A. The pGAL-ArgSec7 construct for expression of the destabilizing Arg-Sec7p mutant. B. Growth curves of the pGAL-SEC7 strain on glucose (solid circles) or galactose (open circles), compared to wild-type yeast (squares) are shown. C. Viability of pGAL-SEC7 yeast after growth on glucose. Equal aliquots of cells shifted to glucose were plated onto glucose or galactose. The number of colonies reflect cell viability at 16.5 and 21h after shut-off of Arg-Sec7p expression.

pared to wild type yeast (Fig. 2A). Thus, even in galactose, Arg-Sec7p was less stable. Arg-Sec7p pools were nearly depleted by 8h, while Met-Sec7p was still evident after 24h in glucose. These results indicate that the amino-terminal residue significantly affected Sec7p stability, in accordance with the N-end rule.

Sec7p function is required for ER-to-Golgi protein traffic. Previous experiments indicated a role for Sec7p in ER-to-Golgi traffic in addition to intra-Golgi transport. The effect of Sec7p depletion was therefore tested on yeast protein traffic *in vivo.* The trafficking of CPY, a soluble vacuolar hydrolase, can be monitored by post-translational modifications as the protein accesses enzymes during transit through specific pathway compartments (9, 24, 25). As shown in Fig. 3A, normal kinetics for CPY maturation were observed in wild-type yeast and the pGAL-SEC7 strain grown in galactose. CPY translocated into the ER appears as a 67 kDa, core glycosylated p1 species. The core oligosaccharides are elaborated in the cis-Golgi compartments by the addition of one to two mannoses in the α1,6 linkage. Further outer chain carbohydrate modifications that occur in subsequent Golgi compartments include mannoses attached in α1,2 and α1,3 linkages to yield the 69 kDa p2 form (9). Upon arrival at the vacuole, p2-CPY is processed to mCPY, the mature 61 kDa form. Thus, CPY traffic was maintained at normal rates in pGAL-SEC7 yeast grown in galactose, despite 5-fold lower steady-state Sec7p levels.

In contrast to the normal profile, CPY immunoprecipitated from radiolabeled lysates of pGAL-SEC7 cells cultured in glucose was recovered as p1-CPY ER species, with some aberrant modification of the CPY intermediates appearing as p1* and m* forms at later chase times (Fig. 3A). Similar results were obtained in *sec7*^{ts} yeast shifted to 37°C. The absence of the p2-CPY intermediate in *sec7* mutants indicates that the aberrant p1* and m* species arose from precocious activity by the α1,6 mannosyltransferases and processing proteases trapped together with the p1-CPY intermediate. This explanation is supported by the observation that the p1* and m* species lacked mannoses added in the α1,3 linkage normally found on p2- and mature CPY that has properly transited the pathway (data not shown). Hence, these results suggest that p1-CPY was trapped in a pre-Golgi compartment upon loss of Sec7p function.

The transport of Gas1p, a glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein (18), was also evaluated. The Gas1p precursor in the ER is a 105 kDa GPI-anchored precursor with N- and O-linked core oligosaccharides. This 105 kDa form was previously characterized as an ER intermediate by accumulation in *sec12* and *sec18* mutant yeast at the restrictive temperature (18, 26). Gas1p maturation in the cis-Golgi resulted in the appearance of a 125 kDa form, as seen in pulse-chase analysis of Gas1p in wild-type and pGAL-SEC7 cells cultured in galactose (Fig. 3B). In contrast, the 105 kDa form accumulated in pGAL-SEC7 in glucose and in *sec7*^{ts} yeast at the restrictive temperature. Hence, the accumulation of membrane-anchored Gas1p duplicated results obtained with the soluble CPY precursor, in that these substrates were trapped in the pathway as pre-Golgi intermediates.

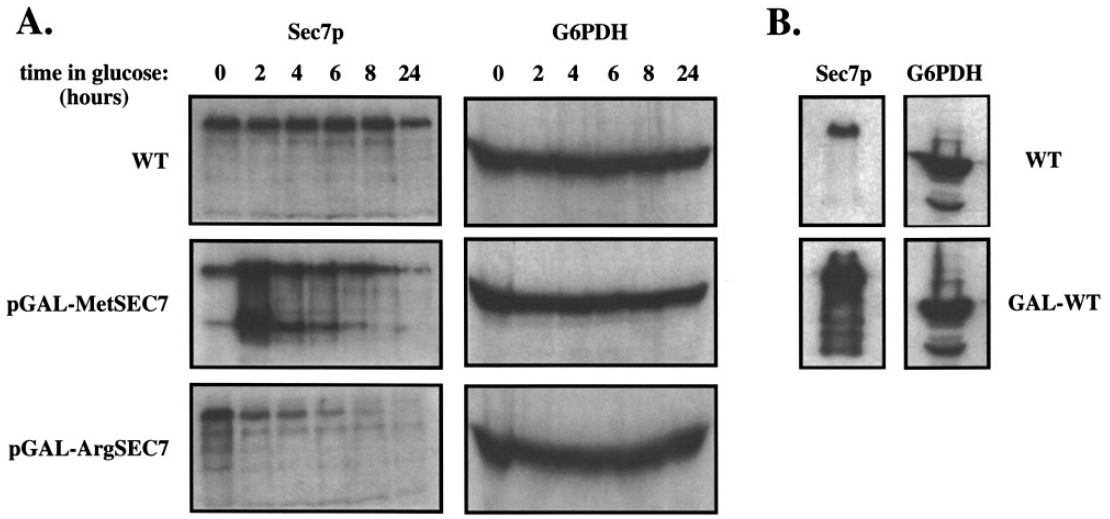

FIG. 2. Sec7p obeys the N-end rule for protein stability. A. Shown are immunoblots of lysates from wild type (WT) cells or yeast expressing the stabilizing Met-Sec7p mutant (pGAL-*MetSEC7*) or the destabilizing Arg-Sec7p mutants (pGAL-*ArgSEC7*) as the sole source of Sec7p expression. Cells were harvested at the indicated times after switch to glucose media. Sec7p levels (left panels) were normalized by the levels of glucose 6 phosphate dehydrogenase (G6PDH, right panels) in the lysate. B. Wild type yeast (WT) and yeast where the wild type SEC7 gene is under galactose promoter control (GAL-WT) were grown on galactose and lysates were blotted for steady state levels of Sec7p (left panels) normalized to G6PDH (right panels).

Immuno-EM labeling of proteins accumulated in sec7 mutants. EM analysis was performed with *sec7-4*^{ts} yeast as the effects due to Sec7p depletion in the conditional pGAL-SEC7 mutant closely paralleled those obtained with the *sec7*^{ts} cells, yet Sec7p inactivation is more rapidly established in the *sec7*^{ts} mutant. EM analysis of the *sec7*ts mutant revealed exaggerations of nucleus-associated and peripheral ER cisternae (Fig. 4). The signature *sec7* mutant stacks of flattened saccules were seen (e.g. Fig. 4B,C), but this exaggerated compartment did not dominate the EM images, as previously reported (10–12).

To investigate the lumenal composition of the exaggerated organelles in *sec7*^{ts} yeast, immuno-labeling was carried out. Invertase is a soluble secreted protein whose expression is induced in the absence of glucose. Pulse-chase analysis in *sec7*^{ts} yeast showed that an underglycosylated invertase was retained within the cell (9). Immunogold localization of invertase in *sec7*^{ts} demonstrated labeling in the lumen of peripheral ER cisternae underlying the plasma membrane (Fig. 4A). In some cells, invertase labeling was observed in cylindrical sheet-like structures with connections to the flattened stacks of membrane cisternae.

Do these exaggerated organelles in *sec7*^{ts} yeast comprise ER, intermediate or Golgi compartments? Proteins that possess a KDEL/HDEL tetrapeptide sequence at their carboxyterminus (e.g. Kar2p/BiP, protein disulfide isomerase) are localized to the ER by retention and retrieval mechanisms (e.g. 27). Attempts to label wild-type yeast with the affinity purified HDEL antisera failed to reveal any specific signals (data not shown). Immuno-gold localization of HDEL-containing proteins in *sec7*^{ts} yeast surprisingly revealed that labeling was restricted to distinct domains of the nucleus-associated and peripheral ER membranes (Fig. 4 C,D). No HDEL antibody labeling of the exaggerated membrane stacks was observed. However, labeling of the abundant HDEL-containing proteins was not evident throughout the ER, but only in distended regions exaggerated by Sec7p inactivation.

DISCUSSION

We have engineered an N-end rule destabilizing mutant of the yeast *SEC7* gene under control of the *GAL10* galactose promoter. Sec7p depletion in pGAL-SEC7 or inactivation in *sec7*^{ts} strains causes cell growth arrest and blocks the traffic of soluble CPY and GPI-linked Gas1p membrane protein in the secretory pathway. The diagnostic post-translational modifications on proteins accumulated in *sec7* mutants reveals a pre-Golgi block in traffic (9, 25–30).

The characterization of a Sec7p requirement in ER-to-Golgi traffic *in vivo* is supported by results from cell-free transport assays. The introduction of Sec7p antibodies to the transport reaction disrupts ER to Golgi traffic *in vitro* (14). Sec7p-coated transport vesicles are immuno-isolated from the Sec7p antibody treated reaction, and the radiolabeled α-factor substrate cargo is recovered as the core-glycosylated ER species. ER to Golgi transport is also blocked in the cell-free assay when wild type cytosolic proteins are substituted by cytosol from *sec7* mutants (7). Thus, the biochemical

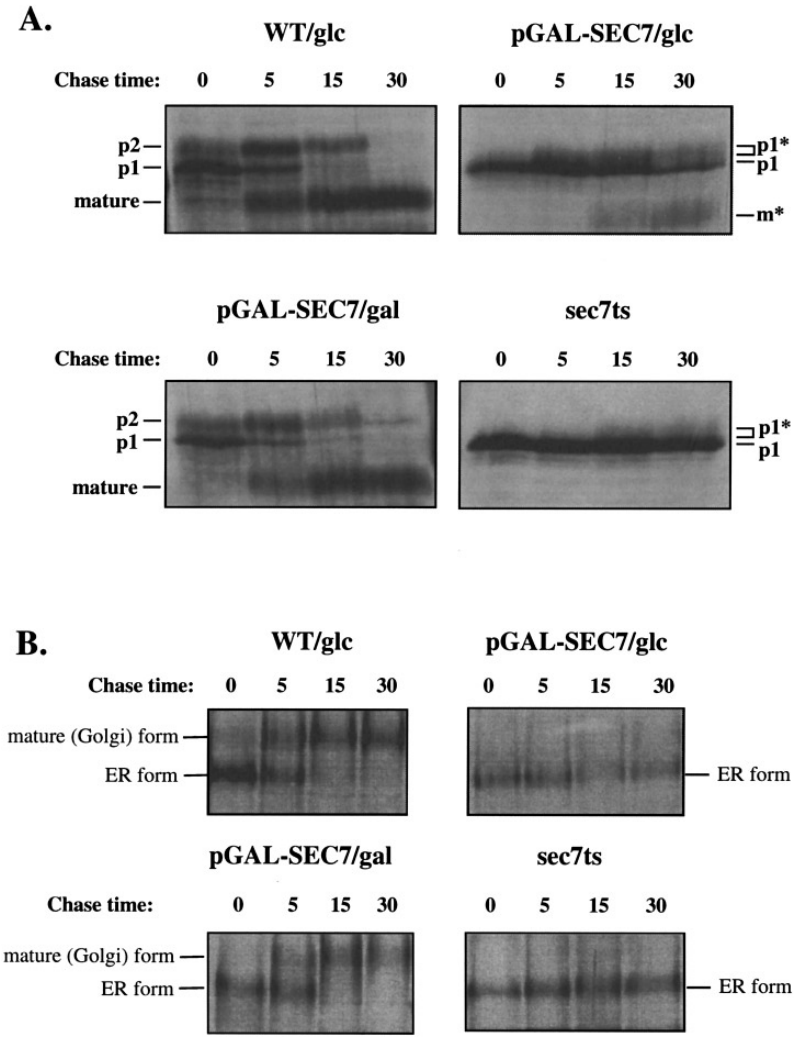

FIG. 3. CPY and Gas1p traffic was disrupted in sec7 mutants. A. Pulse-chase analysis of CPY traffic. Wild type (WT), pGAL-SEC7 and *sec7*^{ts} (AFY80) strains were grown for 16h in the relevant medium. Harvested cells were pulse labeled for 7 minutes in either glucose (glc) or galactose (gal) containing medium, then chased for the indicated times. CPY was immunoprecipitated from cell lysates and resolved on SDS-gels. B. The samples for Gas1p immunoprecipitation were prepared as described in panel A.

evidence supports an assignment for Sec7p in ER-to-Golgi traffic.

The N-end rule works for Sec7p. The destabilizing Arg-Sec7p in pGAL-SEC7 yeast grown on galactose is present at ~5-fold lower levels than wild type Sec7p, yet the mutant grows with normal rates. Arg-Sec7p drops to undetectable levels 8h after shifting the mutant strain from galactose to glucose, yet growth rate is affected only after 13h. This suggests that wild type levels of Sec7p significantly exceed cellular requirements for normal growth. Further, the effect of the N-end rule signature amino acid was highlighted by comparing Sec7p levels with the stabilizing Met residue or the destabilizing Arg residue in the engineered mutants. Both steady state levels in galactose and protein turnover after shift to glucose were dramatically affected. These results emphasize the value of utilizing the N-end rule to affect otherwise stable proteins.

Sec7p is found in both soluble and membrane-bound fractions (4, 6, 7). Fractionation experiments with Arg-Sec7p indicate that the membrane-bound form is more refractory to turnover than the cytosolic Arg-Sec7p pool (data not shown). We are evaluating the consequences of Sec7p depletion on the stability of the proteins normally complexed with Sec7p in the cytosol (6, 15, 31). Thus, use of the pGAL-SEC7 strain will facilitate dissection of the biochemical role for Sec7p and its associated proteins in vesicular traffic.

Sec7p defects affect both ER and Golgi morphology. Support for the hypothesis that the most proximal requirement for Sec7p function is at a pre-Golgi stage of the pathway comes from immuno-EM analysis of the

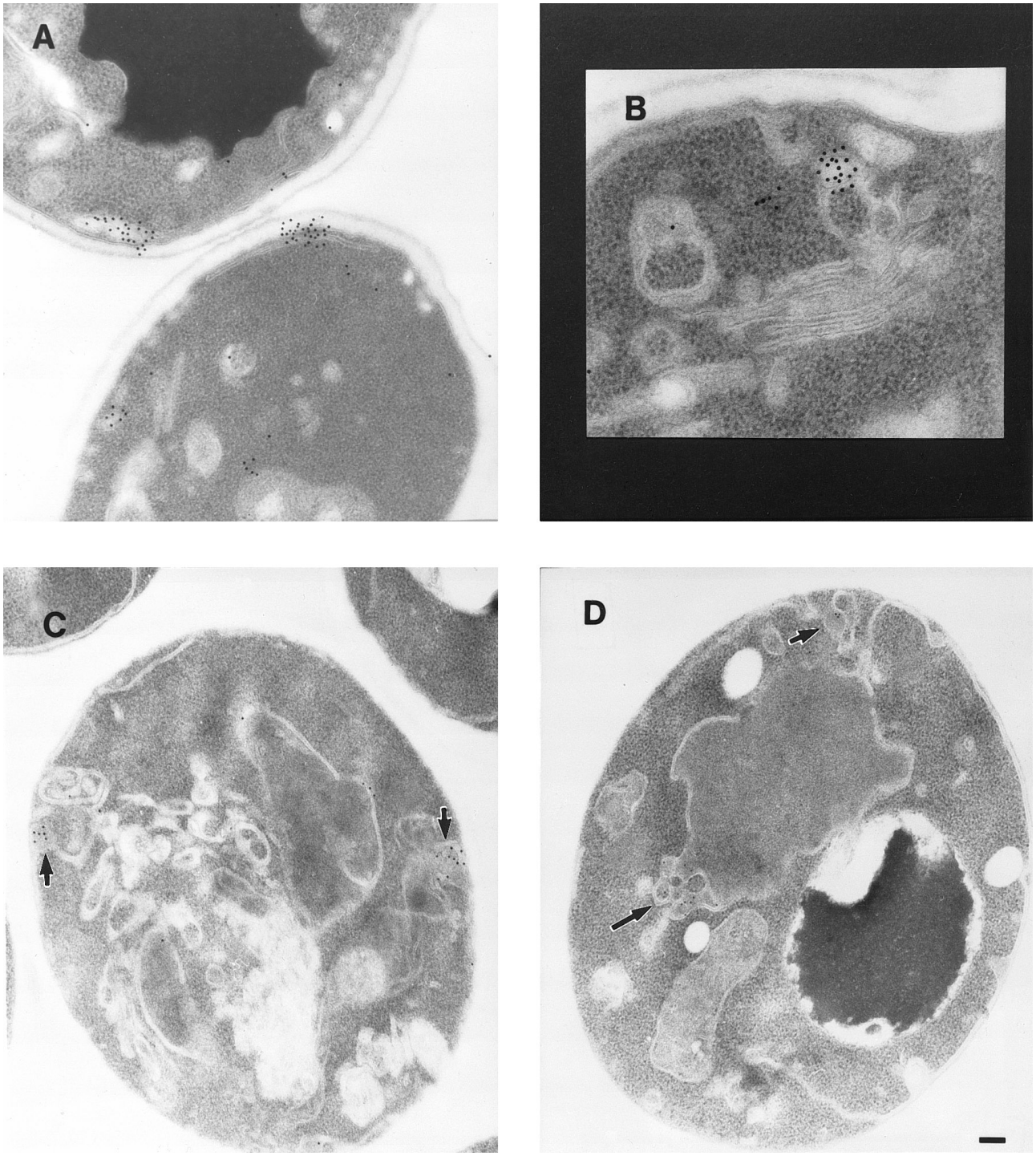

FIG. 4. Immuno-EM labeling of luminal proteins in sec7^{ts} yeast at the restrictive temperature. The top panels are representative examples of immunogold labeling with invertase antibodies accumulated in *sec7*^{ts} yeast at the restrictive temperature. The bottom panels represent immunogold labeling of HDEL-bearing proteins with affinity-purified anti-HDEL peptide antibodies. Note labeling of membrane protuberances (arrows).

sec7^{ts} strain. In Fig. 4, exaggerated organelles seen at the restrictive temperature include protuberances of nucleus-associated and peripheral ER cisternae, membrane stacks, and cylindrical sheet-like structures that resemble Berkeley bodies (first described in (8)). Previous EM studies with *sec7*^{ts} mutants did not emphasize exaggeration of ER compartments, since they were not as obviously affected (8, 10–12). The elegant EM work

from Rambourg et al. revealed the striking and predominant stacks of Golgi cisternae when *sec7*^{ts} yeast were incubated at 37°C, even after 7.5 minutes (12). One principal difference of the *sec7* mutants in this study is the strain background. Earlier EM studies had been performed with *sec7* strains that apparently harbored a suppressor mutation in the strain background, which is eliminated by genetic outcrossing (9, 31). The suppressor masked the ER-to-Golgi defect without influencing the later Golgi traffic blocks. Therefore, in the earlier *sec7*^{ts} mutants, the Golgi phenotype would be more evident than an ER block, while both phenotypes are observed in the present images.

Proteins are sorted into staging areas in sec7^{ts} *mutants.* We observe immunolabeling of both invertase and HDEL-containing proteins in the exaggerated organelles in *sec7*^{ts} yeast (Fig. 4). The HDEL motif, like the mammalian KDEL sequence for protein retrieval, is found at the C-terminus of numerous luminal ER proteins (32). The nuclear apposition of the membrane protruberances favors the interpretation that these protuberances constitute regions of the ER. Immuno-EM of wild type yeast with HDEL antibodies failed to reveal any labeling (E.v.T., unpublished observations), despite the fact that ER localization of the HDEL-containing Kar2p is readily observed by immuno-EM using antibodies to a different segment of the polypeptide (33).

What could be the significance of the HDEL labeling in *sec7*^{ts} yeast, and why is it apparently restricted to these protuberances rather than throughout the ER? The protuberances may represent an ER subdomain, like transitional elements in mammalian cells, where post-ER destined proteins are sorted from the general ER milieu (2, 28, 34). While ER luminal proteins are retained by an HDEL-independent mechanism, proteins with the HDEL motif exit the ER at low levels in wild type yeast (27). In the ER, departing HDEL proteins are separated from the general population. Normally, these HDEL-containing proteins are retrieved from intermediate or Golgi compartments, presumably in association with the HDEL receptor, Erd2p (27, 35). Defects in the retrieval system result in the secretion of HDEL-containing proteins from the cells (19, 27, 35). Binding of the HDEL receptor at distal compartments for retrieval back to the ER obscures the HDEL epitope, thus interfering with immuno-labeling. In *sec7* mutants, the vesicle staging area may be grossly distorted if budding is prevented. The HDEL epitope is no longer shielded from antibody recognition by its receptor or by the general ER retention machinery. This would explain the immunolabeling of the HDEL motif on proteins in these membrane protuberances.

Interestingly, disruption of ER to Golgi transport by overproduction of the Sec12p membrane protein also results in the appearance of membrane protruberances from the nucleus-associated ER, like those seen in *sec7*^{ts} mutants. Immuno-EM of HDEL-containing proteins (Kar2p/BiP and protein disulfide isomerase) and a protein destined for the vacuole, proteinase A, showed labeling in these "BiP bodies" as well as throughout the ER (36). Thus, two independent traffic defects exhibit ER protuberances when vesicle budding is blocked.

An alternate explanation is that these protuberances represent the yeast equivalent of the mammalian intermediate compartment in ER-to-Golgi traffic. The definition of an intermediate compartment in yeast is presently obscure, as it depends on the identification of activities distinct from either the ER or the Golgi (2, 37).

In summary, Sec7p is recruited onto membranes during vesicle assembly, although its biochemical role may be normally encountered after vesicle budding (e.g. uncoating and/or the recruitment of molecules required for targeting) but certainly prior to membrane fusion with the target organelle (15). We propose that the aberrant function of Sec7p in the *sec7*^{ts} strains prevents vesicle budding from the ER, leading to the accumulation of proteins in vesicle staging areas, and causing aberrant dilation of the affected regions. Furthermore, we conclude from *in vivo* and *in vitro* work that the most proximal requirement for Sec7p function is in ER-to-Golgi traffic.

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